IKZF1 deletion is an independent predictor of outcome in pediatric acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol

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IKZF1 deletion is an independent predictor of outcome in pediatric acute lymphoblastic leukemia treated according to the ALL-BFM 2000 protocol

Running title: IKZF1 deletions in pediatric ALL

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Abstract

IKZF1 gene deletions have been associated with a poor outcome in pediatric precursor B-cell acute lymphoblastic leukemia. To assess the prognostic relevance of *IKZF1* deletions for patients treated on Berlin-Frankfurt-Münster Study Group trial ALL-BFM 2000, we screened 694 diagnostic acute lymphoblastic leukemia samples by Multiplex Ligation-dependent Probe Amplification. Patients whose leukemic cells beared *IKZF1* deletions had a lower 5-year event-free survival (0.69±0.05 vs. 0.85±0.01; $p < 0.0001$) compared to those without, mainly due to a higher cumulative incidence of relapses (0.21±0.04 vs. 0.10±0.01; $p=0.001$). Although *IKZF1* deletions were significantly associated with the *P2RY8-CRLF2* rearrangement, their prognostic value was found independent from this association. Thus, *IKZF1* deletion is an independent predictor of treatment outcome and strong candidate marker for integration in future treatment stratification strategies on ALL-BFM protocols.

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Introduction

Approximately 20% of children with acute lymphoblastic leukemia (ALL) still suffer from relapse and may benefit from improved risk stratification and adapted treatment strategies. In this context, the influence of *IKZF1* gene aberrations on therapeutic outcome has received much attention in recent investigations. *IKZF1* encodes for Ikaros, a zinc-finger transcription factor family member, which functions sequence-specific in transcriptional regulation and chromatin remodeling and is required for the development of all lymphoid lineages[1-3]. Somatic aberrations of *IKZF1* have recurrently been observed in precursor B-cell ALL (pB-ALL) - most frequently in those carrying a BCR/ABL rearrangement - and have been shown to confer a poor treatment outcome[4-13]. However, aberrations of *IKZF1* were also found to associate with additional genetic alterations recurrently observed in childhood ALL, such as *JAK* mutations and translocations involving the chemokine receptor like factor 2 (*CRLF2)* gene[4,7,14-18].

Juxtaposition of *CRLF2* to the *IgH@* enhancer or the *P2RY8* promoter lead to elevated expression of wild-type *CRLF2* in ALL and has been shown to exert a negative prognostic impact on outcome[17,18]. However, the prognostic relevance seems to be treatment-dependent and it is not clear yet whether the prognostic impact of *IKZF1* is independent of these concurrent lesions[19].

In the present study, we assessed the prognostic role of *IKZF1* deletions in a large cohort of 694 pediatric ALL patients treated according to the ALL-BFM 2000 protocol.

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Design and Methods

Patients

In accordance with institutional review board regulations, clinical samples were obtained from children with ALL before treatment. The study was approved by the institutional review board of the Hannover Medical School, Hannover, Germany, and informed consent obtained from patients and/or their legal guardians in accordance with the Declaration of Helsinki. Diagnostics, risk group assignment, and treatment were performed according to the ALL-BFM 2000 protocol[20-22]. Risk group stratification (standard risk, SR; intermediate risk, IR; high risk, HR) was mainly based on minimal residual disease (MRD) analysis after induction (time point 1, TP1) and consolidation therapy (TP2)[20]. SR patients were MRD-negative on both TP and HR patients had MRD levels of $\geq 10^{-3}$ at TP2. MRD-IR patients had positive MRD detection at either one or both time points but at a level of $<10^{-3}$ at TP2. Patients with prednisone poor-response (PR; ≥ 1000 leukemic blood blasts / µl on treatment day 8) or induction failure (≥5% leukemic blasts in the bone marrow, BM) or positivity for chromosomal translocations t(9;22)(q34;q11) or t(4;11)(q21;q23) or their molecular equivalents (*BCR/ABL* or *MLL/AF4* rearrangement) were stratified into the HR group, independent of MRD.

Consecutively enrolled patients from the ALL-BFM 2000 study population with sufficient spare leukemic DNA available were included in our present study (Supplementary Table 1). BM or peripheral blood specimens had to contain more than 80% blasts, as assessed morphologically before gradient centrifugation.

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Multiplex ligation-dependent probe amplification (MLPA) analysis

Recently, it was shown that MLPA is ideal for rapid high-throughput testing of large cohorts with a view to establish incidence and prognostic significance of certain gene aberrations[23]. MLPA analysis was performed using the MLPA SALSA kit P335-A3 ALL-*IKZF1* (MRC-Holland, Amsterdam, The Netherlands, www.mlpa.com) according to the manufacturer's protocol. The assay includes probes for each of the eight exons of the *IKZF1* gene and is able to detect deletions of the whole gene as well as all types of focal intragenic deletions. Selected exons of the genes *EBF1, CDKN2A/B, PAX5, ETV6, BTG1, RB1* and the *PAR1* region *(*approx. 230 kBp downstream of *SHOX*, *CRLF2, CSF2RA* and *IL3RA)* are also covered. The *P2RY8-CRLF2* fusion can be identified through assessment of *CSFRA* and *IL3RA* deletions. Fragment analyses were performed on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) and data analyzed using Peak Scanner v1.0 and Coffalyser v9.4 software. Relative copy number was calculated after intra-sample normalization against control fragments and inter-sample normalization against control samples (healthy blood donor DNA). A ratio of 1±0.3 represents a normal copy number of 2, ratios <0.7 and <0.3 represent heterozygous and homozyous deletions, respectively, while ratios >1.3 indicate amplifications. Ratios for all fragments were visually inspected for plausibility and outliers included/excluded where appropriate. In a separate cohort of 25 patients (23 with *IKZF1* deletion), we compared the results from Affymetrix SNP 6.0 arrays with MLPA kit P335 (*Online Supplementary Figure 1*). All 23 deletions that were known from SNP 6.0 arrays were also detected by MLPA, in 19 cases with exact overlap concerning the deleted exons. In three cases not all exons were called correctly

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by MLPA, probably due to subclonality of the deletions. In one additional case, the deletion that was found in exon 3 by MLPA was detected in intron 3 on the SNP 6.0 array due to the location of SNP markers on the array. Comparison of the data obtained by the MLPA kit P335-A3 and the MLPA kit P202-A1 could be undertaken in 78 out of the 84 cases with *IKZF1* deletions and only in one case the deletion was not detected using the MLPA kit P202, due to subclonality. Visual inspection of the results did not allow reliable detection of the lesion in P202 due to a higher background noise. (Online *Supplementary Figure 2*).

Statistical analysis

Event-free survival (EFS) was calculated from the date of diagnosis to last follow-up or to the first event (no complete remission [CR] as event on day 0, relapse, secondary malignancy, or death of any cause). Overall survival (OS) was calculated from the date of diagnosis to date of death by any cause. Rates were calculated according to Kaplan-Meier and compared by log-rank test. Cumulative incidence of relapse (CIR) functions were constructed by the method of Kalbfleisch and Prentice and compared by the Gray test. Cox regression was used to calculate the hazard ratio for an event mediated by *IKZF1* deletions. Proportional differences between patient groups were analyzed by Chi^2 or Fisher's exact tests. Statistical analyses were carried out using the SPSS statistical package (IBM, Chicago, IL, USA) and two-sided P-values below 0.05 were considered to be statistically significant.

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Results and Discussion

Genetic aberrations in *IKZF1* were screened in diagnostic specimens of 694 ALL patients and *IKZF1* deletions were detected in 84 out of 694 samples (12%) (Table 1). This frequency is within the range of abundances reported in other cohorts before[7,9,13]. Twenty-nine of these deletions covered the whole gene (35%), while 55 were focal deletions (65%). As described previously, focal lesions detected were mainly Δ4-7 (23 cases, 42%) and Δ2-7 (4 cases, 7%) deletions (Table 1 and Supplementary Figure 2)[6,8,9,14,23]. Δ4-7 and Δ2-7 deletions represent deletions of exons 4-7 and exons 2-7 respectively. All deletions are visualised in *Online Supplementary Figure 2*.

Comparing leukemic and clinical characteristics of patients with and without *IKZF1* deletion, no significant differences were observed concerning sex, age or presence of an *MLL/AF4* rearrangement (Table 2). There was a positive association of *IKZF1* deletion with higher white blood cell counts (WBC) at diagnosis (p=0.01), pB-ALL immunophenotype (p = 0.01), positivity for a *BCR/ABL* rearrangement (p=0.03), as well as negativity for an *ETV6/RUNX1* rearrangement (p<0.01). Of interest, only five out of 15 *BCR/ABL*-positive cases harbored a deletion, which contrasts previously published data describing the presence of *IKZF1 deletions* in about 80% of *BCR/ABL*-positive ALL.[5,6,8].

Most studies published so far focused on pB-ALL, and we also see a significant association of *IKZF1* deletions with the pB-ALL phenotype. However, our cohort incorporated a total of 116 T-ALL patients, 6 of which showed an *IKZF1* deletion (5.2%). Out of the six T-ALL *IKZF1*-deleted patients, one was treated in the SR group, while the

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others were HR patients. The SR patient is in CR, as are two of the HR patients, while the other three died (only one after a relapse).

Whereas no significant association of *IKZF1* status with PR was observed, the presence of *IKZF1* deletions was significantly more frequent in MRD-HR patients as compared to MRD-IR and MRD-SR patients ($p < 0.01$). Five-year EFS was significantly lower in patients with an *IKZF1* deletion compared to those without (0.69±0.05 vs. 0.85±0.01; $p < 0.0001$) (Figure 1A). This difference was mainly attributable to a significantly higher CIR in *IKZF1*-deleted patients (0.21±0.04 vs. 0.10±0.01; p=0.001) (Figure 1B). This effect was most pronounced in the IR group, where the 5-y EFS was 0.71±0.07 in *IKZF1*-deleted patients compared to 0.84±0.02 in those without (p=0.008). The corresponding CIR were 0.26±0.07 and 0.10±0.02, respectively (p=0.0002) (Figures 1D and 1E). Fifty percent of all events and 13 of 19 relapses (68%) occurred in the IR group. No significant differences in EFS were seen in SR patients (deletion-positive vs. negative patients: 5-y EFS 0.85±0.08 vs. 0.92±0.02, p=0.42; CIR 0.08±0.02 vs. 0.05±0.05, p=0.54). While in HR-patients EFS was 0.46±0.11 in the *IKZF1*-deleted vs. 0.69±0.05 in the non-deleted group (p=0.03), the CIR did not differ significantly (0.25±0.10 vs. 0.15±0.04, p=0.28).

Five-year OS was also significantly reduced in patients with *IKZF1* deletions compared to patients without it (0.82±0.04 vs. 0.92±0.01, $p = 0.003$). The differences were, however, not significant when analyzing the data according to the current risk groups: (SR: deletion-positive vs. negative patients: 5-y OS 0.99±0.01 vs. 1.00, p=0.52; IR: 0.86±0.05 vs. 0.93±0.01, p=0.09; HR: 0.56±0.11 vs. 0.75±0.04, p=0.06). This suggests

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that patients with *IKZF1* deletions who subsequently relapse are in part still treatment sensitive in the relapse situation.

In a Cox regression analysis considering the additional variables sex, initial WBC, MRD, and presence of the *MLL/AF4*, *BCR/ABL* and *ETV6/RUNX1* rearrangement, *IKZF1* deletions provided independent prognostic information (hazard ratio for an event 2.28, 95% confidence interval, CI, 1.44-3.60; $p < 0.001$, *Online Supplementary Table 2*).

IKZF1 deletions have been described to be associated with rearrangements of *CRLF2* and, recently, we have shown that the *P2RY8-CRLF2* rearrangement is associated with a poor outcome in patients treated according to the ALL-BFM 2000 protocol[17]. In the study presented here, we confirmed a significantly higher number of *IKZF1* deletions in patients positive for the *P2RY8-CRLF2* rearrangement (13.1%) in comparison to those negative (2.6%; $p<0.001$). To explore whether *IKZF1* has prognostic relevance independent of *CRLF2* status, we first excluded *CRLF2*-rearranged patients from the multivariate analysis accounting for the strong association of the two factors (*IKZF1* deletion and *P2RY8-CRLF2* rearrangement), and the resulting co-linearity in the model. In this analysis, *IKZF1* deletions still provided independent prognostic information (hazard ratio for an event 1.94, 95% CI 1.19-3.16; $p = 0.008$). Cox regression analysis including both factors resulted in a hazard ratio of 2.08 (95% CI 1.32 – 3.28, $p=0.002$) for *IKZF1*-deletions and a hazard ratio of 2.04 (95% CI 0.94 – 4.41, $p=0.07$) for *P2RY8-CRLF2* (*Online Supplementary Tables 3-4*).

Comparing clinical and biological characteristics of patients with whole and focal gene deletions, no significant differences for sex, WBC, immunophenotype, involvement of the central nervous system (CNS), presence of *BCR/ABL*, *MLL/AF4* or *ETV6/RUNX1*

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rearrangements, PR, MRD and final risk stratification were observed (data not shown). Patients with focal deletions were significantly older compared to patients with whole gene deletions (>10 years: 38% vs. 10%; *p*=0.01). There were no significant differences in 5-year EFS or CIR neither comparing whole and focal deletions nor comparing the different types of *IKZF1* deletions (*data not shown*).

IKZF1 deletions were repeatedly reported to confer an increased risk of relapse and poor outcome in selected cohorts[6,9] and, recently, in an unselected cohort of 131 pB-ALL cases[12,13]. Here, we present data on the prognostic relevance of *IKZF1* in a large set of nearly 700 patients uniformly treated on the MRD-based ALL-BFM 2000 protocol and were able to show that *IKZF1* status exerts additive value as a prognostic factor - especially in the IR group in which still the majority of relapses occurs. Therefore, the assessment of *IKZF1* status may contribute to a molecular-based stratification algorithm aiming at improving outcome for children suffering from ALL. However, more work has to be done to elucidate the molecular differences between *IKZF1*-deleted leukemias of patients who relapse and those who don't. From our perspective, this is a prerequisite for an application of the *IKZF1* status in clinical decision making.

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Authorship and Disclosures

PD performed laboratory work, data analysis and drafted the manuscript; BM and JPB made initial observations; MZ performed statistical analysis; AM and DS contributed to data interpretation and AM co-ordinates the ALL-BFM study; AS, RK and CRB performed MRD analysis; JH performed cytogenetic investigations; WDL performed immunophenotyping of patient samples; MS is leader of the ALL-BFM study and contributed to data interpretation. MSt and GC designed the study, contributed to data analysis and interpretation as well as writing of the paper. The authors report no potential conflicts of interest.

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Tables and Figures

Table 1. MLPA analysis of *IKZF1* deletions in 694 patients with pediatric ALL.

***IKZF1* status**	**Patients, n**	**%**
Not-deleted	610	88
Deleted[1]	84	12
Whole gene	29	35
Focal	55	65
Δ4-7 deletion	23	42
Δ2-7 deletion	4	7
Other focal deletion	28	51

[1]Deletions are divided into whole gene and focal deletions while focal deletions are divided into three subgroups. Δ4-7 and Δ 2-7 deletions represent deletions of exons 4-7 and exons 2-7 respectively, following the nomenclature of NCBI. All deletions are visualised in Supplementary Table 3.

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Table 2. Patient characteristics and response to treatment according to *IKZF1* deletion status in 694 pediatric patients with ALL.

Feature	***IKZF1* not deleted n (%)**	***IKZF1* deleted n (%)**	***p*[1]**
Number of patients	610 (100)	84 (100)	
Sex			0.91
Male	353 (57.9)	48 (57.1)	
Female	257 (42.1)	36 (42.9)	
Age at diagnosis (years)			0.42
1 – 9	460 (75.4)	60 (71.4)	
± 10	150 (24.6)	24 (28.6)	
Presenting WBC count ($x10^{10}$/L)			0.01
< 1	237 (38.9)	19 (22.6)	
1 – 4.99	222 (36.4)	35 (41.7)	
5 – 9.99	83 (13.6)	14 (16.7)	
≥ 10	68 (11.1)	16 (19.0)	
Prednisone response[2]			0.99
Good	529 (86.7)	71 (84.5)	
Poor	78 (12.8)	10 (11.9)	
No information	3 (0.5)	3 (3.6)	

DOI: 10.3324/haematol.2011.056135

MRD[3]			<0.01
MRD-SR	226 (37.0)	23 (27.4)	
MRD-IR	241 (39.5)	35 (41.7)	
MRD-HR	32 (5.2)	15 (17.9)	
No information	111 (18.3)	11 (13.0)	
Immunology			0.01
Non-T-ALL	496 (81.3)	76 (90.5)	
T-ALL	110 (18.0)	6 (7.1)	
No information	4 (0.7)	2 (2.4)	
ETV6/RUNX1			<0.01
Negative	423 (69.3)	76 (90.5)	
Positive	141 (23.1)	4 (4.75)	
No information	46 (7.6)	4 (4.75)	
BCR/ABL1			0.03
Negative	590 (96.7)	78 (92.9)	
Positive	10 (1.6)	5 (5.9)	
No information	10 (1.6)	1 (1.2)	
MLL/AF4			0.99
Negative	570 (93.4)	81 (96.4)	
Positive	1 (0.2)	0	
No information	39 (6.4)	3 (0.6)	

DOI: 10.3324/haematol.2011.056135

Final risk group			0.06
SR	219 (35.9)	21 (25.0)	
IR	288 (47.2)	42 (50.0)	
HR	103 (16.9)	21 (25.0)	

[1]X^2 test comparing *IKZF1* normal and deleted groups, patients with no information excluded from test; [2]good: less than 1000 leukemic blood blasts / µl on treatment day 8, poor: more than 1000 / µl.
[3]MRD risk groups[17]: MRD-SR: TP1+2 negative, MRD-IR: TP1 and/or TP2 $<10^{-3}$, MRD-HR: TP2 $\geq 10^{-3}$.

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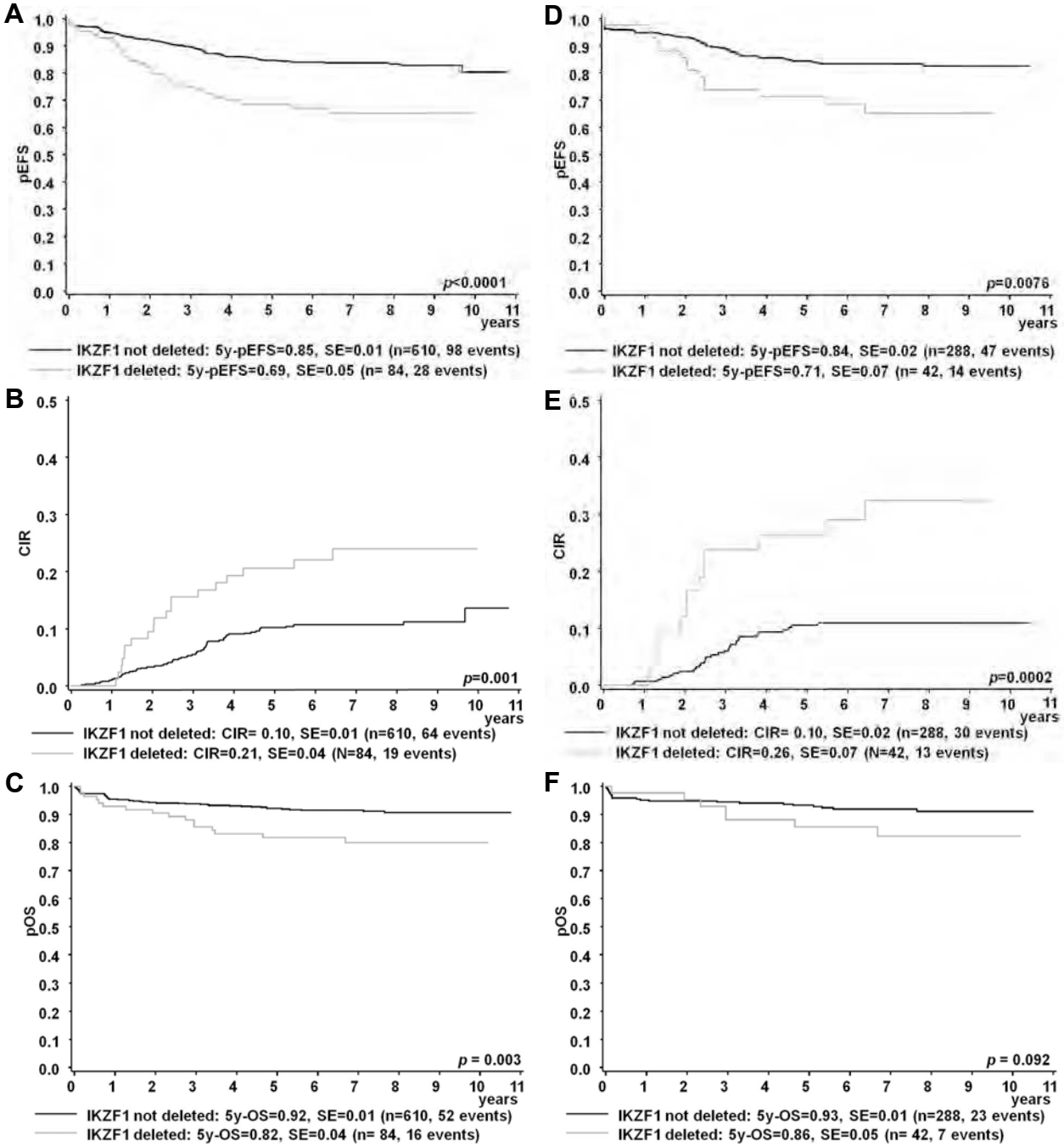
Figure legend

Figure 1. Treatment outcome of patients with pediatric ALL comparing patients with and without *IKZF1* deletions. For the whole cohort of 694 patients the Kaplan-Meier estimates of event free survival (EFS) at 5 years (A), cumulative incidence of relapse (CIR) at 5 years (B) and overall survival (OS) at 5 years (C) are displayed. Also, for the intermediate risk group, EFS (D), CIR (E) and OS (F) are shown.

DOI: 10.3324/haematol.2011.056135

Figure 1.



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Online Supplementary Appendix

Online Supplementary Figure 1. Comparison of results from Affymetrix SNP 6.0 arrays and MLPA kit P335, regarding *IKZF1* deletions, in 25 selected patients.

Online Supplementary Figure 2. Results for all 84 *IKZF1* deleted patients as analysed with MLPA kit P335 and validation with MLPA kit P202.

Online Supplementary Table 1. Patient characteristics and response to treatment in the study cohort of 694 pediatric patients with ALL, in comparison to the remaining ALL-BFM 2000 patient cohort that was not studied.

Online Supplementary Table 2. Multivariate cox regression analysis for event-free survival, including all 694 patients.

Online Supplementary Table 3. Multivariate cox regression analysis for event-free survival, excluding 27 *P2RY8-CRLF2* positive patients from the cohort.

Online Supplementary Table 4. Multivariate cox regression analysis for event-free survival, including *P2RY8-CRLF2* status in the model.

DOI: 10.3324/haematol.2011.056135

Online Supplementary Table 1. Patient characteristics and response to treatment in the study cohort of 694 pediatric patients with ALL, in comparison to the remaining ALL-BFM 2000 patient cohort that was not studied.

Feature	**Cohort not studied (%)**	**Study cohort (%)**	**P[1]**
Number of patients	2133 (100)	694 (100)	
Sex			0.20
Male	1170 (54.9)	400 (57.6)	
Female	963 (45.1)	294 (42.4)	
Age at diagnosis (years)			0.90
1 – 9	1603 (75.2)	520 (74.9)	
± 10	530 (24.8)	174 (25.1)	
Presenting WBC count ($x10^{10}$/L)			<0.0001
< 1	1091 (51.1)	257 (37.0)	
1 – 4.99	666 (31.2)	256 (36.9)	
5 – 9.99	175 (8.2)	97 (14.0)	
≥ 10	201 (9.4)	84 (12.1)	
Prednisone response[2]			0.005
Good	1920 (90.0)	601 (86.7)	
Poor	189 (8.9)	87 (12.5)	
No information	24 (1.1)	6 (0.8)	
MRD[3]			0.57

DOI: 10.3324/haematol.2011.056135

MRD-SR	687 (32.2)	249 (35.9)	
MRD-IR	847 (39.7)	276 (39.8)	
MRD-HR	132 (6.2)	46 (6.6)	
No information	467 (21.9)	123 (17.7)	
Immunology			0.03
Non-T-ALL	1787 (83.8)	572 (82.5)	
T-ALL	280 (13.1)	116 (16.6)	
No information	66 (3.1)	6 (0.9)	
ETV6/RUNX1			0.80
Negative	1488 (69.8)	498 (71.7)	
Positive	449 (21.0)	146 (21.1)	
No information	196 (9.2)	50 (7.2)	
BCR/ABL			0.58
Negative	2053 (96.2)	668 (96.2)	
Positive	39 (1.8)	15 (2.2)	
No information	41 (2.0)	11 (1.6)	
MLL/AF4			0.23
Negative	1979 (92.8)	651 (93.8)	
Positive	10 (0.5)	1 (0.1)	
No information	144 (6.7)	42 (6.1)	
Final risk group			0.003
SR	667 (31.3)	240 (34.6)	
IR	1167 (54.7)	331 (47.7)	

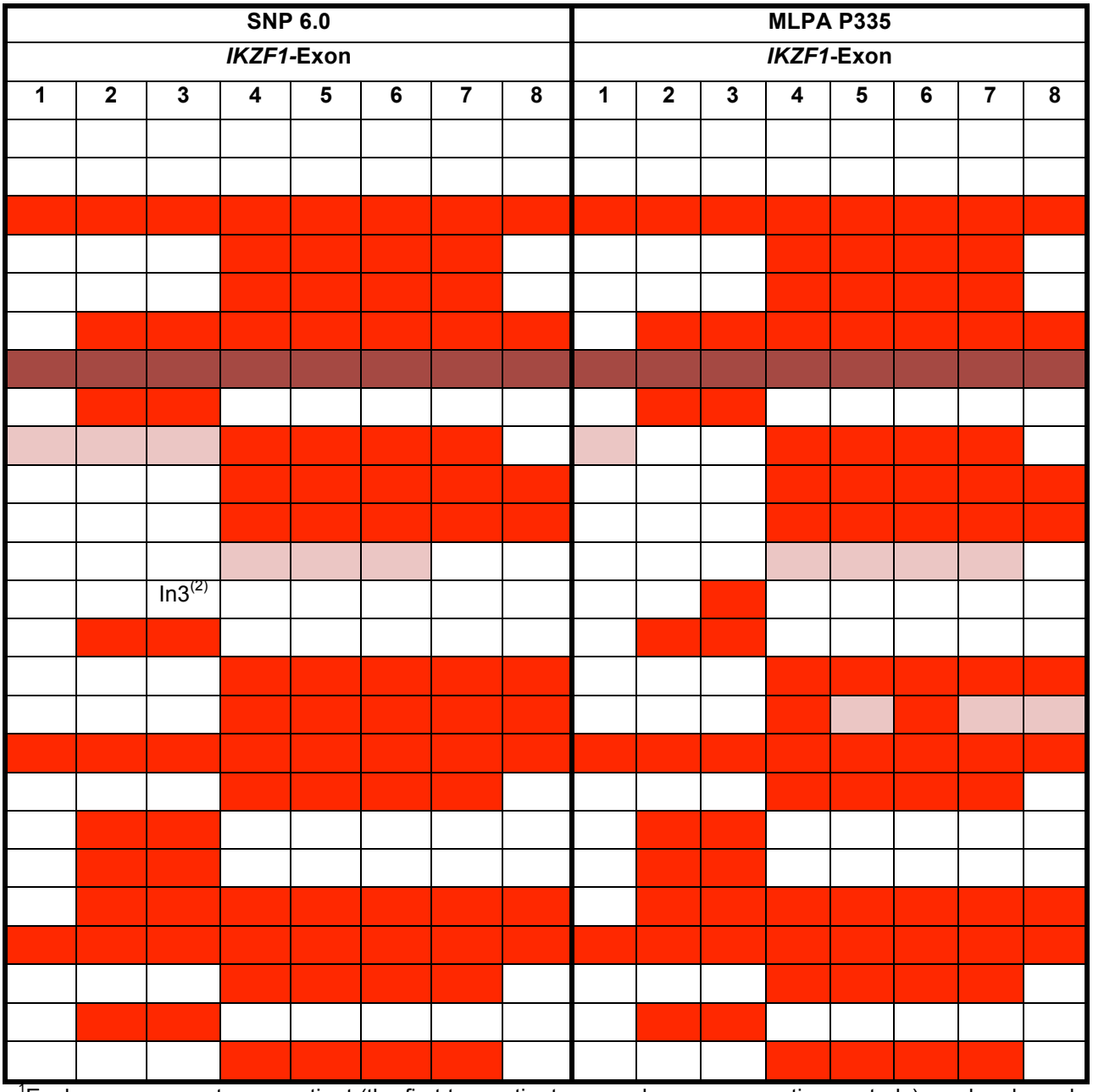
DOI: 10.3324/haematol.2011.056135

HR	299 (14.0)	123 (17.7)	

[1]X^2 test comparing study cohort and patients not studied, patients with no information excluded from test;
[2]good: less than 1000 leukemic blood blasts / µl on treatment day 8, poor: more than 1000 / µl.
[3]MRD risk groups[17]: MRD-SR: TP1+2 negative, MRD-IR: TP1 and/or TP2 $<10^{-3}$, MRD-HR: TP2 $\geq 10^{-3}$.

DOI: 10.3324/haematol.2011.056135

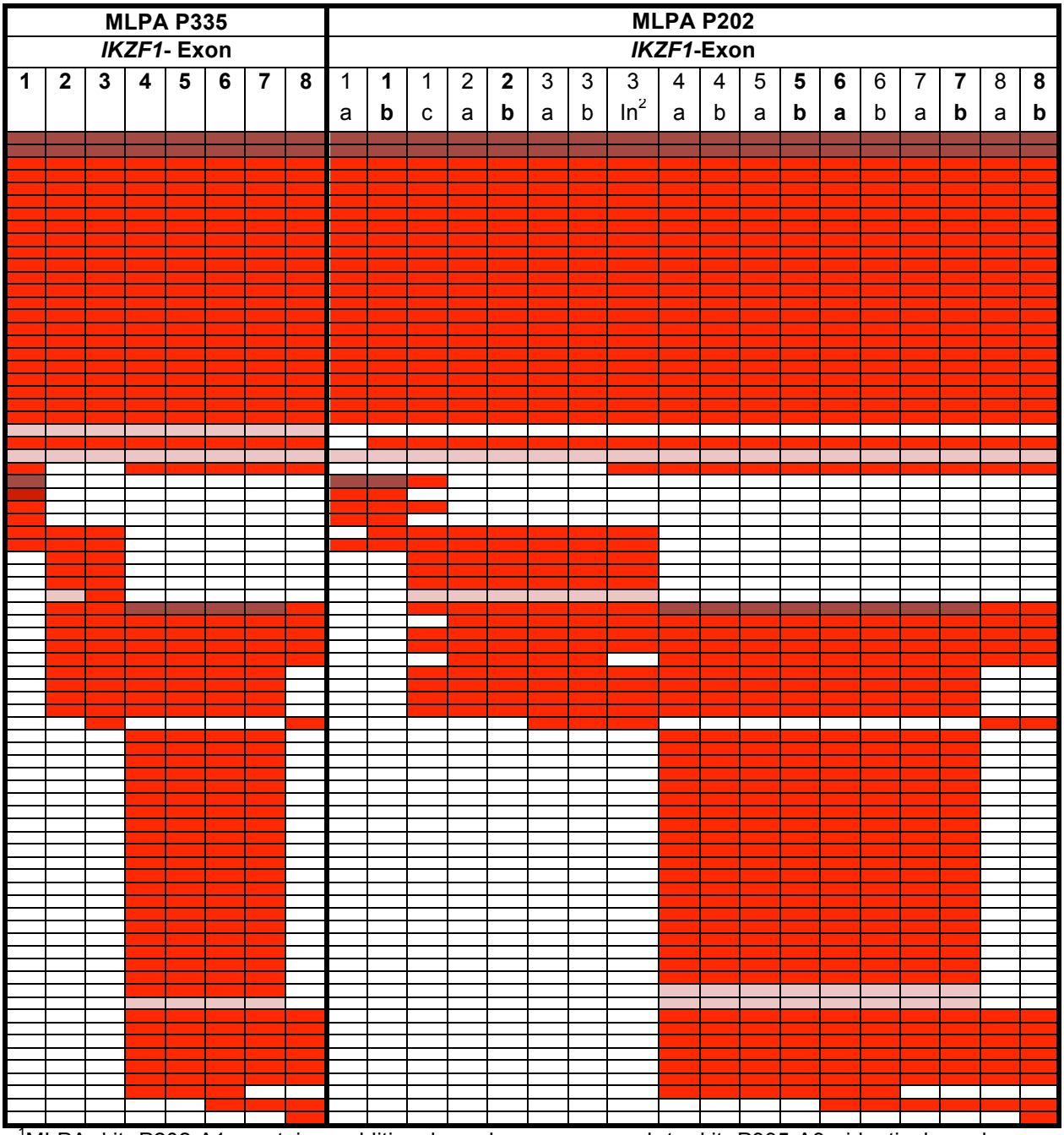
Online Supplementary Figure 1. Comparison of results from Affymetrix SNP 6.0 arrays and MLPA kit P335, regarding *IKZF1* deletions, in 25 selected patients[1].



[1]Each row represents one patient (the first two patients were chosen as negative controls), each coloured lane represents a deletion in the respective exon. Light red deletions are heterozygous, dark red deletions are homozygous and light pink deletions are subclonal.
[2]Deletion on SNP 6.0 was found in Intron 3.

DOI: 10.3324/haematol.2011.056135

Online Supplementary Figure 2. Results for 78 *IKZF1* deleted patients as analysed with MLPA kit P335 and validation with MLPA kit P202[1].



[1]MLPA kit P202-A1 contains additional markers, compared to kit P335-A3, identical markers are highlighted in bold. Each row represents one patient, each coloured lane represents a deletion in the respective exon. Light red deletions are heterozygous, dark red deletions are homozygous and light pink deletions are subclonal. In one subclonal sample, results were discordant between P335 and P202, as indicated. Of the six un-validated samples four had a Δ1-8, one a Δ4-7 and one a Δ5-7 deletion in P335.
[2]In: Intron.

DOI: 10.3324/haematol.2011.056135

Online Supplementary Table 2. Multivariate cox regression analysis for event-free survival, including all 694 patients.

Feature	Hazard ratio	95% CI	*p*
IKZF1	2.28	1.44 – 3.60	< 0.0001
Sex	0.91	0.62 – 1.33	0.62
SR	0.56	0.34 – 0.93	0.03
HR	1.91	1.20 – 3.03	0.006
ETV6/RUNX1	1.00	0.58 – 1.73	0.99
Immunology	0.99	0.59 – 1.67	0.97
WBC ≥ 100,000	1.46	0.89 – 2.39	0.14

Online Supplementary Table 3. Multivariate cox regression analysis for event-free survival, excluding 27 *P2RY8-CRLF2* positive patients from the cohort.

Feature	Hazard ratio	95% CI	*p*
IKZF1	1.94	1.19 – 3.16	0.008
Sex	0.91	0.61 – 1.34	0.62
SR	0.58	0.34 – 0.99	0.05
HR	1.98	1.24 – 3.17	0.004
ETV6/RUNX1	1.03	0.59 – 1.79	0.92
WBC ≥ 100,000	1.53	0.93 – 2.51	0.09

DOI: 10.3324/haematol.2011.056135

Online Supplementary Table 4. Multivariate cox regression analysis for event-free survival, including *P2RY8-CRLF2* status in the model.

Feature	**Hazard ratio**	**95% CI**	***p***
IKZF1	2.08	1.32 – 3.28	0.002
P2RY8-CRLF2	2.04	0.94 – 4.41	0.07
Sex	0.90	0.62 – 1.32	0.591
SR	0.54	0.33 – 0.91	0.02
HR	2.07	1.31 – 3.28	0.002
ETV6/RUNX1	1.07	0.60 – 1.85	0.81
WBC ≥ 100,000	1.44	0.89 – 2.32	0.14